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Efficient control of gene expression by a tetracycline-dependent transactivator in single *Dictyostelium discoideum* cells

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Abstract

We established a tetracycline-regulated gene expression system that tightly controls expression of genes in *Dictyostelium discoideum*. The control elements are contained in two plasmid vectors, one being an integrated plasmid encoding a chimeric tetracycline-controlled transcriptional activator protein (tTA_s). The second component is an extrachromosomal plasmid harboring the gene of interest preceded by an inducible promoter. This promoter contains a tetracycline-responsive element, which is the binding site for tTA_s. Tetracycline prevents tTA_s from binding to the tetracycline-responsive element, rendering the promoter virtually silent. In the absence of tetracycline, tTA_s binds to its target sequence and strongly induces gene expression. The kinetics of activation and repression of the system were monitored using luciferase as a reporter. The results reveal efficient inhibition of gene expression by low concentrations of tetracycline and an induction of gene expression by several orders of magnitude within a few hours after removal of tetracycline. Green fluorescent protein (GFP) provided information about the effects of modulation of the tetracycline concentration on gene expression, at the single cell level, using fluorescence activated cell sorting (FACS). We also report that not all cells in a clonal population express the reporter gene. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Green fluorescent protein; Fluorescence activated cell sorting; Inducible promoter; Luciferase

1. Introduction

Dictyostelium discoideum is a soil amoeba having signal transduction pathways similar to those of mammalian cells, leading to chemotaxis and cell differentiation. Furthermore, it shows mammalian-like glycosylation and architecture (Slade et al., 1997; Parent and Devreotes, 1999). Since *D. discoideum* cells can be easily transformed and cultured, and are haploid during the major part of their life-cycle, rapid screening of mutants can be performed for studying the role of endogenous genes in *D. discoideum* (Kim et al., 1997).

In addition, *D. discoideum* can be employed to express functional heterologous proteins (Slade et al., 1997) and for mutant screening purposes of complex recombinant human glycoproteins (Linskens et al., 1999). Modulation of the level of expression of endogenous genes in a time-controllable fashion is a valuable tool for analyzing the phenotypes associated with mutations in genes. Tight control of gene expression is especially advantageous for potential cytotoxic proteins. However, previous attempts to regulate gene expression in vegetatively growing cells of *D. discoideum* have met with limited success, due to the lack of tightness of control (Blusch et al., 1992; Dingermann et al., 1992; Wetterauer et al., 1993).

The tetracycline-controlled inducible expression system, originally described by Gossen and Bujard (1992), provides tight control of gene expression in mammalian cells by low, non-toxic levels of tetracycline (Tc). Briefly, this system employs a Tc-controlled transcriptional activator (tTA), that is negatively regulated by Tc. This transactivator interacts specifically with an inducible promoter, that controls the transcription of

Abbreviations: A15P, actin15 promoter; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting; G418, Geneticin; GFP, green fluorescent protein; *gfp*⁺, gene encoding GFP; 2H₃T, 2H₃ terminator; *luc*⁺, gene encoding luciferase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tc, tetracycline; *tetO*, Tc-resistance operator; TetR, Tet repressor protein; TRE, Tc responsive element; tTA, Tc-controlled transactivator; VP16, herpes simplex virus protein 16.

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the gene of interest. The transactivator tTA is a fusion of the DNA-binding Tet repressor (TetR) from *Escherichia coli* to the activation domain from herpes simplex virus protein 16 (VP16AD), thereby converting it from a repressor into a eukaryotic transcriptional activator protein (Gossen and Bujard, 1992). The inducible promoter consists of a minimal promoter, preceded by a Tc-responsive element (TRE) encompassing seven copies of a Tc-resistance operator (*tetO*) from *E. coli* (Gossen and Bujard, 1992).

In the absence of Tc, tTA binds specifically to TRE and strongly activates the minimal promoter, leading to transcription of the gene of interest. In the presence of Tc, however, a conformational change in the Tet repressor moiety prevents tTA from binding to the TRE and the promoter is silent (Gossen and Bujard, 1992; Shockett and Schatz, 1996). Gene expression can be gradually reduced by adding increasing amounts of Tc (Gossen and Bujard, 1992).

We have adapted the Tc-controlled expression system for application in *D. discoideum*. A modified transactivator (tTA_s) that is encoded on a vector randomly integrated in the genome is constitutively expressed under control of the complete *D. discoideum* actin15 promoter (Cohen et al., 1986; Hori and Firtel, 1994). An extra-chromosomal response plasmid contained a luciferase or GFP gene fused to a Tc-inducible promoter (TRE-P_{min}) consisting of a minimal *D. discoideum* actin15 promoter preceded by seven copies of *tetO*. We report here our results from experiments expressing firefly luciferase (de Wet et al., 1987) and GFP (Cheng et al., 1996) using this inducible system. While luciferase expression provided information about the kinetics and induction in cells at the population level, FACS enabled us to investigate the regulation of GFP expression at the individual cell level.

2. Materials and methods

2.1. Culture conditions and transformation of *D. discoideum* cells

Cells of strain AX3 were grown in axenic medium essentially as described in Watts and Ashworth (1970), with 10 g/l of glucose instead of 15.4 g/l. *D. discoideum* cells were transformed with circular plasmid DNA by electroporation as previously described (Heikoop et al., 1998; Howard et al., 1988). Depending on the antibiotic-resistance cassette present in the plasmids, the medium was supplemented with 10 mg/l blasticidin S (ICN) and/or 20 mg/l G418 (Gibco). The cell lines obtained after transformation of the transactivator plasmid (see Section 2.2.2) served as host cell lines for the transformation of response plasmid (see Section 2.2.1), containing either a luciferase or a GFP encoding reporter gene (see

Sections 2.2.3 and 2.2.4, respectively). After this second transformation the cells were maintained in the presence of both blasticidin and G418. Tetracycline (Roche) was present at the concentrations indicated in the experiments.

2.2. Construction of plasmids

Most of the plasmids were constructed as described below by multiple cloning steps according to standard methods (Sambrook et al., 1989). Codon preference and translation initiation signals are different in *D. discoideum* (Fey et al., 1995; Firtel and Chapman, 1990; Sharp and Devine, 1989; Vervoort et al., 2000) as compared with those of other organisms. During cloning we adapted sequences to match the preferred usage in *D. discoideum*. *E. coli* XL1-Blue (Stratagene) was used as a host for the construction of all the plasmids mentioned. PCR was performed with *Taq* DNA polymerase (Roche) or the Expand High Fidelity System (Roche). Amplified DNA sequences were analyzed by dideoxy sequencing.

2.2.1. Construction of the response plasmid

Except for the Tc-inducible promoter, the response plasmid MB38 (Fig. 1) contains essentially the same elements as the previously described extrachromosomally maintained plasmid MB12N (Heikoop et al., 1998). In short, the orientation of the blasticidin resistance cassette was reversed. In addition, a double-stranded synthetic oligonucleotide was inserted in the unique *Bgl*II site in order to create a multiple cloning site for the insertion of the gene to be expressed (Fig. 1b). The resulting plasmid, still containing the complete *D. discoideum* actin15 promoter (A15P) in front of the multiple cloning site, was labeled MB33. The inducible promoter TRE-P_{min} was created by multiple cloning steps using pGEMNB, a pGEM7Zf(+) (Promega)-derived plasmid in which we introduced an *Nde*I- and *Bgl*II-site in between the *Bam*HI- and *Hind*II-site of the multiple cloning region. First, the TRE from plasmid pTRE (Clontech), containing seven copies of *tetO*, was cloned in pGEMNB after digestion with *Sma*I and *Xho*I (plasmid MB24). Second, the *Xba*I/*Bgl*II fragment containing the complete A15P region of plasmid BS18.2H₃ (gift from R. Firtel) was subcloned in pGEMNB (plasmid MB26). The minimal *D. discoideum* actin15 promoter (A15P_{min}), consisting of region –127 to –14 of A15P (Cohen et al., 1986; Hori and Firtel, 1994), was isolated from MB26 after digestion with *Acs*I, filling in the overhanging ends using Klenow, and cutting with *Bgl*II. Subsequently, the A15P_{min} fragment was fused downstream of TRE in MB24 cut with *Sma*I and *Bgl*II, to give the Tc-inducible promoter (Fig. 1b). To complete the construction of MB38, the A15P of the expression

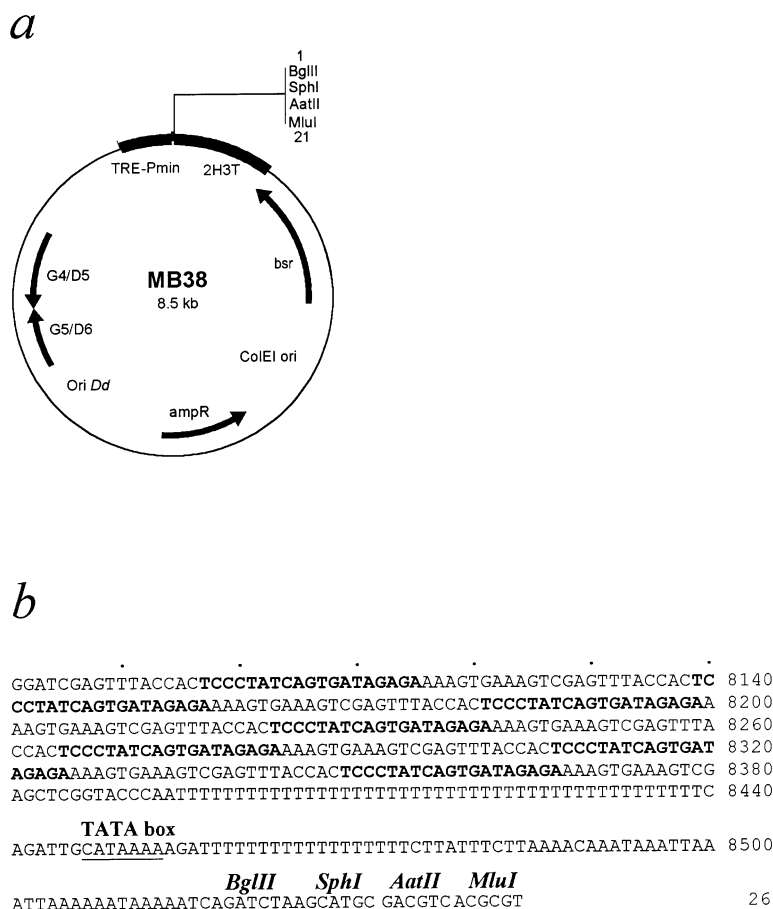


Fig. 1. Response plasmid. (a) Map of the extrachromosomal response plasmid MB38. The plasmid contains sequences for propagation (ColEI ori) and ampicillin resistance (ampR) in *E. coli* (from pBluescript SK), a blasticidin resistance cassette (bsr) consisting of the blasticidin deaminase gene under control of the complete *D. discoideum* actin15 promoter and the *D. discoideum* actin8 terminator, sequences for extrachromosomal maintenance in *D. discoideum* (G4/D5, G5/D6), a *D. discoideum* origin of replication (Ori Dd), and the inducible promoter (TRE-P_{min}) upstream of the *D. discoideum* 2H₃T terminator (2H₃T). Four unique restriction sites are present for insertion of the gene to be expressed. (b) Sequence of the inducible promoter and the multiple cloning region of MB38. Bases 8086–8392 correspond to the Tc-responsive element (TRE) containing seven copies of the *tetO* operator sequence (shown in bold letters). Bases 8393–8506 contain the *D. discoideum* actin15 minimal promoter (P_{min}), corresponding to the –127 to –14 region of the *D. discoideum* actin15 promoter. The position of the *D. discoideum* TATA box is indicated, as well as the unique restriction sites for insertion of the gene to be expressed. Note that the cloned insert must have an initiation codon.

cassette was replaced by the Tc-inducible promoter TRE-P_{min}.

2.2.2. Construction of the transactivator plasmid

The transactivator plasmid MB35 (Fig. 2) is based on the integrating *D. discoideum* vector BS18.2H₃ conferring G418-resistance to *D. discoideum* host cells. It contains the tTA_s encoding gene (*tTA_s*) under control of A15P and the 2H₃ terminator (2H₃T). *tTA_s* was adapted for use in *D. discoideum* as follows. First, the 621 bp *TetR* part of *tTA_s* was obtained by PCR with plasmid pTet-Off (Clontech) as a template, using 5'-GGGGAAGATC TAAAAAATGT CTAGATTAGA TAAAAGTAAAGTG-3' as the 5'-primer and 5'-GC-AAGGGATC CACTTTCACA TTAAAGTTG-3' as the 3'-primer. Five As were introduced upstream of the

initiation codon (underlined), and *Bgl*II- and *Bam*HI-sites were included for cloning purposes. Second, the 255 bp 3' part of *VP16AD* in plasmid pTet-Off was amplified by PCR; concomitantly, seven codons in the 5' region from the resulting short *VP16AD* as well as the stop codon (underlined) were replaced by codons preferred by *D. discoideum* (table from Vervoort et al, 2000), using 5'-GGGTTTGATC ACACACTCGT AGACTCTCAA CTGCCCCACC AACCGATGTC AGCCTGGGGG-3' as the 5'-primer and 5'-GC-GCGGGATC CTTAACCACC GTACTCGTCA ATTCC-3' as the 3'-primer. *Bcl*II- and *Bam*HI-sites were introduced for subsequent cloning. Sequence analysis revealed the presence of one silent mutation (nucleotide 768, GGT to GGC) in *VP16AD_s*. Via multiple cloning steps using pGEMNB, the modified activation domain

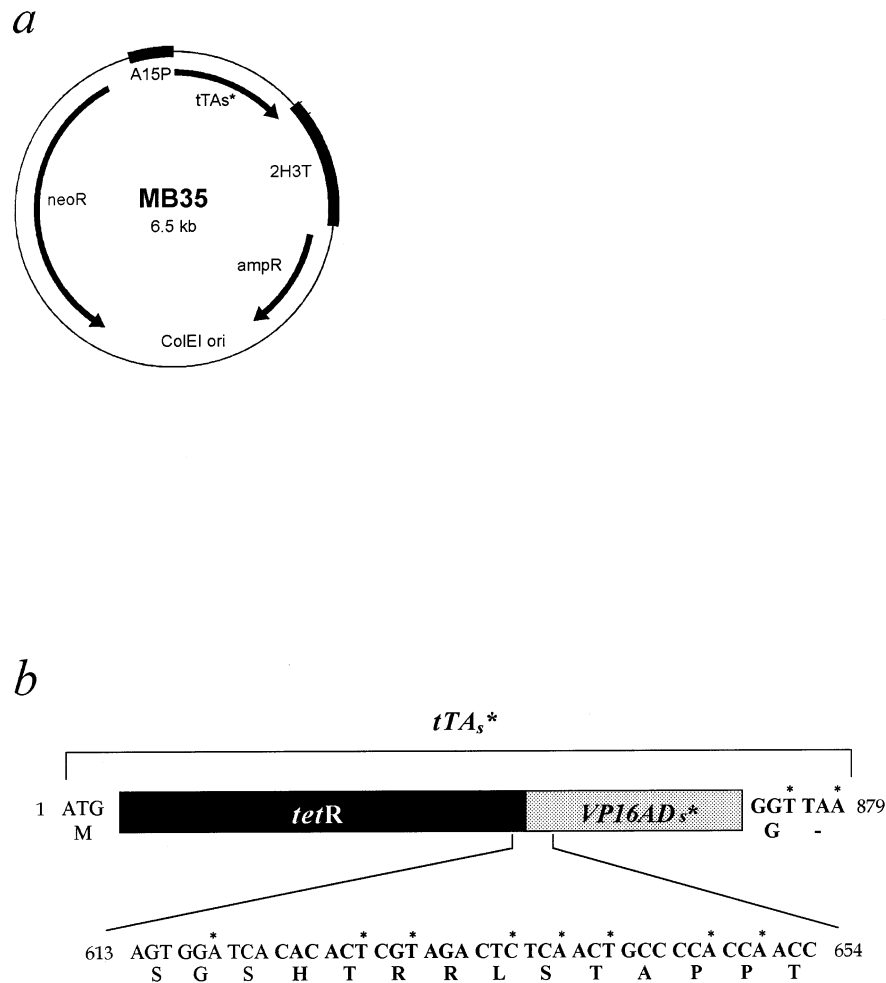


Fig. 2. Transactivator plasmid. (a) Map of the integrating transactivator plasmid MB35. The plasmid contains sequences for propagation (ColEI ori) and ampicillin resistance (ampR) in *E. coli* (from pUC18), a *D. discoideum* actin6 promoter-driven G418 resistance cassette (neoR) and the gene encoding the transactivator (*tTAs**) driven by the complete *D. discoideum* actin15 promoter (A15P) and followed by the *D. discoideum* 2H₃T terminator (2H₃T). (b) Schematic representation of the gene encoding the transactivator (*tTAs**). Bases 1–621 encode the original 207 amino acids of TetR (*tetR*), bases 622–876 the original 85 C-terminal amino acid residues of the VP16 activation domain (*VP16AD_s**). Numbers indicate nucleotide positions in *tTAs**; amino acid residues are presented below the nucleotide sequence. Letters in bold designate bases and amino acid residues of *VP16AD_s**. Nucleotides mutated to create codons adapted to the preferred codon usage in *D. discoideum* are indicated by an asterisk. The gene contains one silent mutation (nucleotide 768) introduced by PCR (not shown).

(*VP16AD_s**) was cloned downstream the modified *TetR* moiety. The resulting *tTAs** was inserted into the *Bgl*II-site of BS18.2H₃.

2.2.3. Construction of luciferase reporter plasmids

The firefly luciferase gene was used as reporter gene (de Wet et al., 1987). Four As were introduced in front of the ATG start codon and six of the first 10 codons were adapted to the preferred codon usage of *D. discoideum* (Sharp and Devine, 1989; Vervoort et al., 2000). For this purpose, a double-stranded synthetic oligonucleotide containing *Bam*HI- and *Nar*I- compatible overhanging ends, sense oligonucleotide: 5'-GATCCAAAA ATGGAAGATG CTAAAAATAT TAAGAAAGGT CCAG-3' (start codon underlined), antisense oligonucleotide: 5'-CGCTGGACCT TTCTTAATAT TTTTAG-

CATC TTCCATTTTTTG-3', was inserted in *Bam*HI/*Nar*I-digested plasmid pGEM-*luc* (Promega). This modified *luc** was subcloned as a *Bam*HI/*Stu*I-fragment downstream of TRE-P_{min} or A15P in the *Mlu*I-(rendered blunt by Klenow) and *Bgl*II-sites of MB38 and MB33, respectively.

2.2.4. Construction of GFP reporter plasmids

The GFP-S65T gene (Cheng et al., 1996) was adapted for usage in *D. discoideum*. In the modified gene (*gfp**) a TAA stop codon was introduced, as well as four As in front of the ATG start codon. Plasmid pS65T-C1 (Clontech) served as template for the amplification of GFP-S65T. The following primers were used: 5'-AGCTTGGATC CAAAAATGGG TAAAGGA-GAAGAAC-3' (start codon underlined) as 5'-primer

and 5'-TGTGTAGATC TTACTTGTAT AGTTCAT-CC-3' as 3'-primer (stop codon underlined), containing a *Bam*HI- and a *Bgl*III-site, respectively, for insertion of *gfp** in the *Bgl*III-sites of MB38 and MB33.

2.3. RNA preparation and hybridization

Cells (1×10^7) were grown on a 9 cm Petri dish and washed once with EB [10 mM sodium phosphate buffer (pH 6.7), 50 mM sucrose] at 22°C. Cells were collected by centrifugation and resuspended in 0.6 ml RLT lysis buffer (QIAGEN). The lysed cells were homogenized by passing them several times through a 0.5×16 mm needle fitted to a syringe. Total cellular RNA was prepared using the RNeasy kit (QIAGEN) following the manufacturer's protocol. For Northern blot analysis, 10 µg of RNA was diluted in RNA gel loading buffer and resolved on a formaldehyde gel. After electrophoresis, RNA was transferred onto a Nytran membrane (Schleicher & Schuell) and cross-linked by UV. Radioactive probes were generated by end-labeling with [γ - 32 P]ATP (Amersham). The synthetic antisense oligonucleotides 5'-GGGAAAACAG CACGTGGAGCATCGTCACCA GCAAAACCGG C-3' and 5'-CGCTGGACCT TTCTTAATAT TTTTAGCATC TTCCATTTTGG-3' served as *actin15* and *luc** probes, respectively. Hybridization was first performed at 50°C with the *luc** probe in 0.5 M Na₂HPO₄/H₃PO₄ (pH 7.2), 1 mM Na₂EDTA, 0.2 mg/ml BSA, 7% SDS. After hybridization, filters were washed 2×15 min in 40 mM Na₂HPO₄/H₃PO₄ (pH 7.2), 1 mM Na₂EDTA, 5% SDS and 4×15 min in 40 mM Na₂HPO₄/H₃PO₄ (pH 7.2), 1 mM Na₂EDTA, 1% SDS at 65°C (*actin15* probe) or 50°C (*luc** probe) quantified using a PhosphorImager (Molecular Dynamics, ImageQuant software). The blot was stripped and re-probed with the *actin15* probe at 65°C. The *luc** hybridization signal was normalized relative to the *actin15* hybridization signal in each sample.

2.4. Luciferase assays

Cells were grown in 24-well multidishes until 50–90% confluency was reached (0.5×10^6 cells), and resuspended in 0.5 ml of EB. After centrifugation, the cells were resuspended in 30 µl of CCLR (Promega) supplemented with $1 \times$ Complete Mini EDTA-free (Roche) protease inhibitor cocktail and 5 mM of the phosphatase inhibitor sodium orthovanadate (ICN) (Rutherford et al., 1997). After incubation at 22°C for 20 min, the cell extracts were frozen at -80°C . Immediately after thawing, 5 µl of cell extracts were mixed with 50 µl of Luciferase Assay Reagent (Promega) in Fluoro Nunc opaque white 96-well plates (Nunc). Luminescence was detected using a FL600 Fluorescence microplate reader (Bio-Tek Instruments). The lamp was turned off and

the emission filter was removed during measurements. Luminescence was quantified using the luminescence of recombinant luciferase (Promega) as a standard. The luciferase activity was normalized to the protein concentration of the cell extract as determined by the method of Bradford (1976).

2.5. Flow cytometry

Expression of *gfp**, displaying excitation and emission maxima at 490 and 511 nm, respectively (Cheng et al., 1996), was analyzed by FACS analysis using an Epics Elite flow cytometer (Coulter). Approximately 3000 or 5000 intact cells were illuminated at 488 nm and fluorescence was detected in the FITC (525/20 nm) channel. Non-specific fluorescence was detected using a 575/30 nm emission filter in the PE channel. The relative fluorescence signal is given in arbitrary units (AU).

Cells were grown on plates until 10–30% confluency, washed $3 \times$ with EB at 22°C, and resuspended in EB at a concentration of $1\text{--}5 \times 10^6$ cells/ml. When cells were cultured in the presence of Tc, the same concentration of Tc was added to EB. Cells were kept on ice until FACS analysis was carried out.

3. Results

3.1. Construction and characterization of the inducible promoter

TRE-P_{min}, the promoter that can be negatively regulated by Tc, was harbored in the extrachromosomally maintained response plasmid MB38. To generate TRE-P_{min}, the enhancer sequences upstream from position -127 of the complete *D. discoideum* A15P (Cohen et al., 1986; Hori and Firtel, 1994) were replaced by a DNA fragment containing seven *tetO* sequences (Gossen and Bujard, 1992) (Fig. 1b). The orientation of TRE-P_{min} was opposite to that of the A15P of the blasticidine resistance cassette (Fig. 1), in order to avoid possible readthrough from the A15P on transcription from TRE-P_{min}. In order to test the residual activity of TRE-P_{min}, luciferase expression driven by TRE-P_{min} was measured in AX3 cells, in the absence of transcriptional activator protein and Tc. Luciferase activity was indistinguishable from the signal measured in AX3 cells lacking a luciferase gene, which is undetectable (below 0.2 ng luciferase/mg protein) (Table 1), whereas the luciferase expression level driven by A15P ranged from 447 to 876 ng luciferase/mg total protein. Similar results were obtained for cells growing in the presence of 2 mg Tc/l (data not shown). These results demonstrate that the minimal promoter region of TRE-P_{min} is silent.

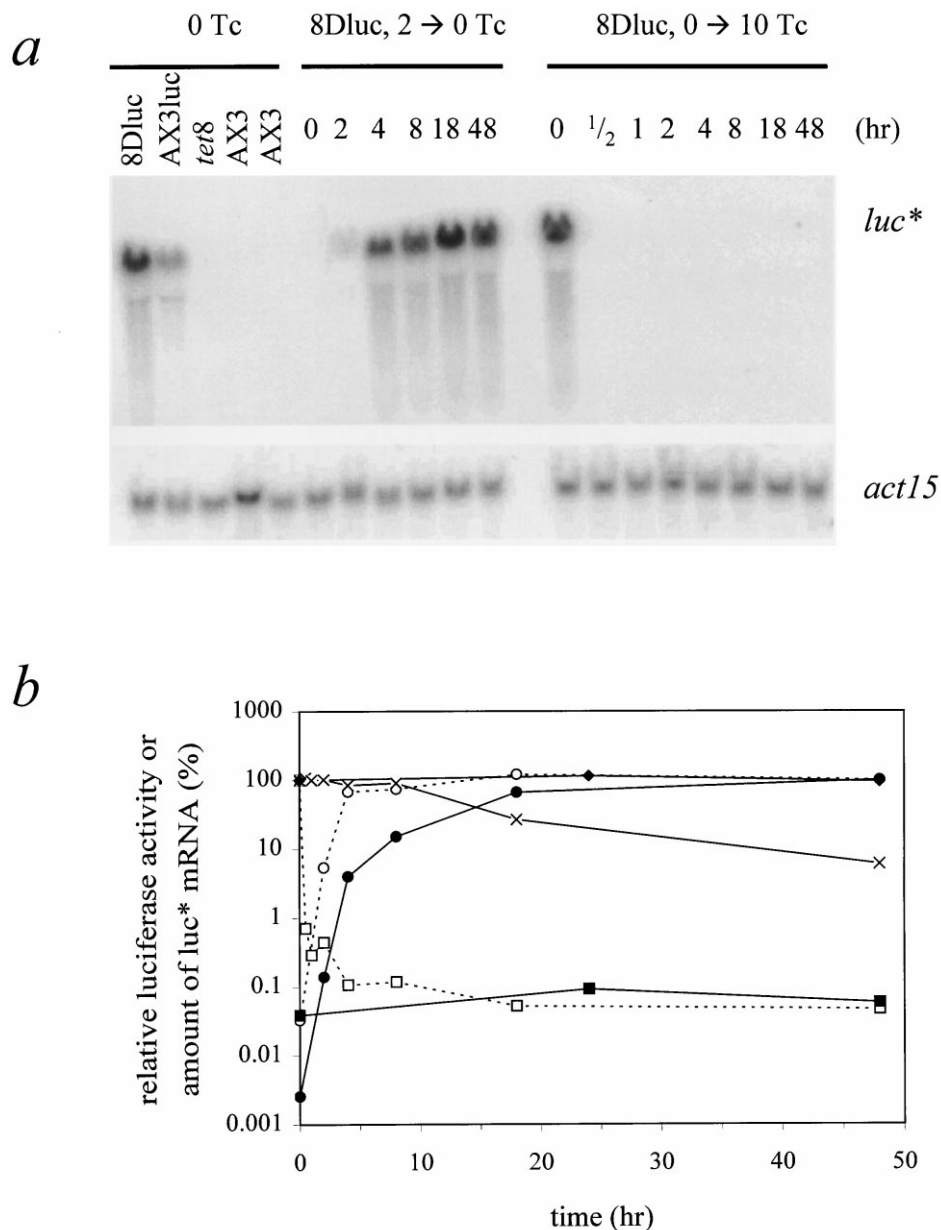


Fig. 3. Time course of Tc action. (a) Northern blot analysis of luciferase mRNA (*luc**) produced by 8Dluc cells, AX3 cells transformed with the A15P/*luc* plasmid (AX3luc) or untransformed (AX3) and *tet8* cells (*tet8*). Total RNA was isolated from cells grown in the absence of Tc for 4 days (0 Tc), after removal of Tc (2→0 Tc) or addition of Tc (0→10 Tc) at different times (h) indicated. The actin15 mRNA (*act15*) is shown as a loading control. (b) Cells of clone 8Dluc were grown on plates in HG5 medium in the absence (diamonds) or presence (2 mg/l) (squares) of Tc. Induction of *luc** mRNA (open circles, dotted line) and luciferase activity (circles) was monitored after removal of Tc at time 0. Reduction of luciferase mRNA (open squares, dotted line) and luciferase activity (×s) was measured after the addition of Tc (10 mg/l) to Tc-free medium at time 0. A relative luciferase activity of 100% corresponds to 958 ng luciferase/mg protein.

3.2. Construction and transactivation potential of *tTA**_s

The transactivators *tTA* and *tTA*_s, originally described by Gossen and Bujard (1992), contain a C-terminal portion of the VP16 activation domain of 128 amino acids (VP16 codons 363–490) or 85 amino acids (VP16 codons 406–490), respectively. When fused to the *E.coli* TetR, both transcriptional activator proteins exhibited essentially the same transactivation

potential (Gossen and Bujard, 1992). During cloning we adapted sequences to match the preferred usage in *D. discoideum*, since codon preference and translation initiation signals are different in *D. discoideum* (Fey et al., 1995; Firtel and Chapman, 1990; Sharp and Devine, 1989; Vervoort et al., 2000) as compared with other organisms. For instance, 17 of VP16 codons 363–405 are used at a frequency lower than 0.6% in *D. discoideum*, which may negatively influence expression.

Table 1
Maximal luciferase activity in the absence of tetracycline^a

Host cell line	Reporter construct	Relative luciferase activity in the absence or presence of Tc (%) ^b									
		Population of clones		Clone A		Clone B		Clone C		Clone D	
		–Tc	+Tc	–Tc	+Tc	–Tc	+Tc	–Tc	+Tc	–Tc	+Tc
AX3	A15P/–	<0.03	n.d.								
AX3	A15P/ <i>luc</i> *	100 ± 7.1	n.d.	79.6 ± 8.9	n.d.	66.2 ± 4.4	n.d.	94.4 ± 15.3	n.d.	15.3 ± 0.9	n.d.
AX3	TRE- <i>P</i> _{min} / <i>luc</i> *	<0.03	n.d.								
<i>tet2</i>	TRE- <i>P</i> _{min} / <i>luc</i> *	45.0 ± 2.6	<0.03	48.0 ± 2.6	<0.03	31.3 ± 0.6	<0.03	129.8 ± 8.9	<0.03	47.2 ± 1.8	<0.03
<i>tet3</i>	TRE- <i>P</i> _{min} / <i>luc</i> *	11.0 ± 3.1	<0.03								
<i>tet5</i>	TRE- <i>P</i> _{min} / <i>luc</i> *	49.5 ± 3.8	<0.03	31.4 ± 6.2	<0.03	29.2 ± 4.2	<0.03	53.7 ± 3.1	<0.03	33.3 ± 1.8	<0.03
<i>tet6</i>	TRE- <i>P</i> _{min} / <i>luc</i> *	35.1 ± 13.8	<0.03								
<i>tet7</i>	TRE- <i>P</i> _{min} / <i>luc</i> *	90.3 ± 18.0	<0.03								
<i>tet8</i>	TRE- <i>P</i> _{min} / <i>luc</i> *	34.1 ± 1.5	<0.03	33.1 ± 2.8	<0.03	26.6 ± 3.2	<0.03	59.0 ± 10.1	<0.03	130.0 ± 25	0.04
<i>tet15</i>	TRE- <i>P</i> _{min} / <i>luc</i> *	5.5 ± 1.7	<0.03								

^a Cell lines expressing *tTA*_s (*tet2* to *tet15*) were clonal with respect to *tTA*_s expression. After transformation of *luc** reporter plasmids, cells from different subclones were grown on plates in HG5 medium with or without Tc (10 mg/l) (+Tc and –Tc, respectively). Presented are average values and standard deviations of two to three independent experiments. n.d., not determined.

^b Luciferase activity was related to the A15P-driven luciferase expression level in AX3 cells (100% = 675 ng luciferase/mg total protein).

We therefore decided to fuse a short activation domain (*VPI6AD*_s), containing codons 406–490 of *VPI6*, to the *TetR* region. The resulting coding sequence of *tTA*_s was expressed constitutively under control of the complete *D. discoideum* A15P (plasmid MB35, Fig. 2).

The capacity of *tTA*_s to activate luciferase expression and of Tc to reduce expression was assessed in different *tTA*_s expressing *tet* cell lines. A slight reduction in growth rate was observed in most of the *tet* cell lines (approximately one doubling per 16 h) as compared with AX3 cells (one doubling per 12 h). Upon transformation with the TRE-*P*_{min}/*luc** response plasmid, cells were grown either in the absence or in the presence of 10 mg Tc/l. As shown in Table 1, in the presence of Tc luciferase activity was not or hardly detectable. In contrast, in the absence of Tc, *tTA*_s strongly activates the inducible promoter. The luciferase expression level ranged from 5% to 130% as compared with the expression driven by the *D. discoideum* complete A15P (Table 1). This variation is likely due to the different integration sites of the transactivator plasmid in the different *tet* cell lines. A variation in expression levels was also observed in individual subclones of the same *tet* cell line after transformation of the TRE-*P*_{min}/*luc** response plasmid. The highest induction factor observed was approx. 3000-fold in *tet8* + TRE-*P*_{min}/*luc** cells, clone D (8Dluc cells), resulting from an induced expression level of 130% and a basal expression level of 0.04% (Table 1). In other clones, the expression level under maximally inducing conditions was at least 500-fold above the basal expression level, which was below 0.03% (0.2 ng luciferase/mg protein).

3.3. Kinetics of activation and inactivation

The 8Dluc cell line, that expressed the highest level of luciferase in the induced state and shows a basal expression level just above the detection limit, was selected for further analysis. 8Dluc cells were grown for several days on Petri dishes in HG5, both in the absence and the presence of Tc. As shown in Fig. 3, removal of Tc (2 mg/l) by rinsing and replacing Tc-containing medium with Tc-free medium resulted in an increase in *luc** mRNA and luciferase activity, reaching 50% of the maximally induced level within 8 h (*luc** mRNA) or 18 h (luciferase activity). Upon addition of Tc (10 mg/l) to fully induced cells, the amount of *luc** mRNA dropped rapidly from 100% to less than 1% of the initial level

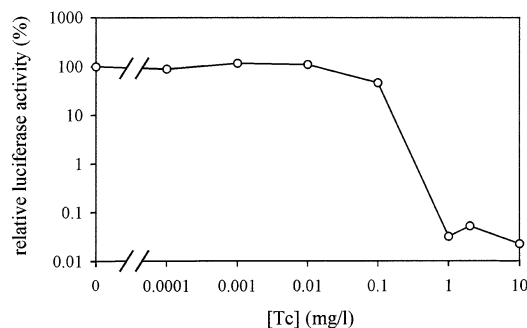
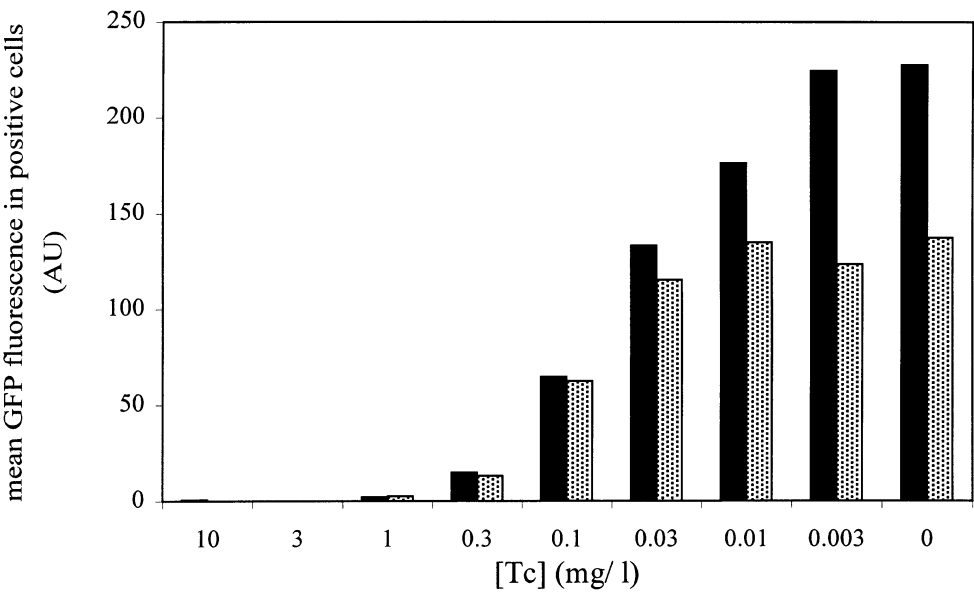


Fig. 4. Dose–response analysis of Tc-regulated *luc** expression. Relative luciferase activity in 8Dluc cells grown on plates in HG5 medium containing different concentrations of Tc as indicated. After 4 days, the cells were assayed for luciferase activity. Average values of two independent experiments are given. A relative activity of 100% corresponds to 958 ng luciferase/mg protein.

a



b

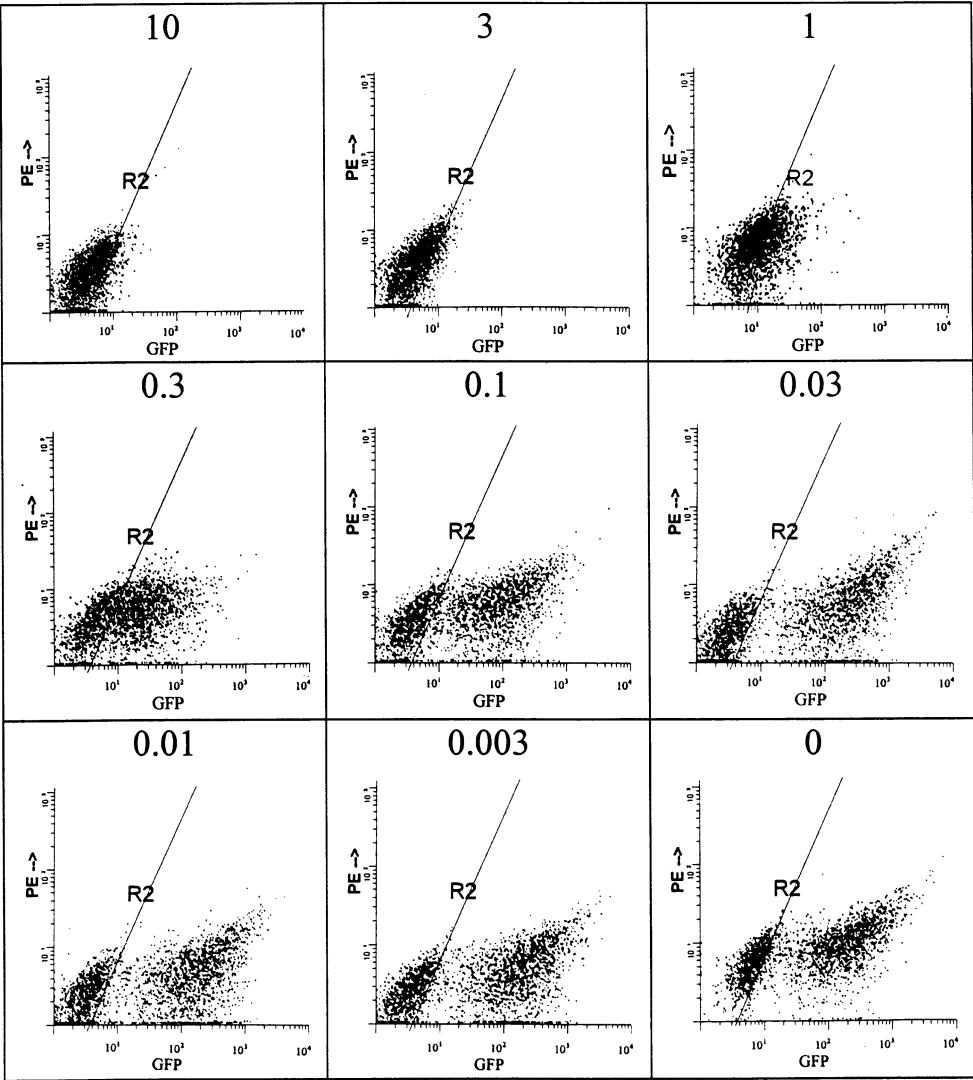


Table 2
Mean GFP fluorescence in GFP-positive cells^a

Host cell line	Reporter construct	Mean fluorescence in GFP-positive cells (AU) and fraction of GFP positive cells (% of total cells)			
		Clone A	Clone B	Clone C	Clone D
AX3	A15P/ <i>gfp</i> *	119 (32%)	136 (29%)	109 (38%)	136 (28%)
<i>tet2</i>	TRE-P _{min} / <i>gfp</i> *	108 (32%)	53 (17%)	44 (11%)	81 (30%)
<i>tet5</i>	TRE-P _{min} / <i>gfp</i> *	101 (33%)	229 (58%)	136 (39%)	157 (63%)
<i>tet8</i>	TRE-P _{min} / <i>gfp</i> *	120 (32%)	92 (21%)	112 (20%)	160 (20%)

^a Cell lines *tet2*, *tet5* and *tet8* were clonal with respect to *tTA*_s* expression. Cells of subclones obtained after transformation of *gfp** reporter plasmids were grown on plates in Tc-free HG5 during 4 days. GFP expression in individual cells was analyzed using FACS. GFP fluorescence was the mean fluorescence signal in GFP positive cells in arbitrary units (AU), determined using excitation at 488 nm and a 525/20 nm emission filter.

within 30 min. This provided us with an estimation of the biological half-life of *luc** mRNA of less than 6 min. However, after 48 h (about three doubling times) approx. 6% of the maximal luciferase activity was still present per cell. Presumably this luciferase was synthesized prior to the addition of Tc, and diluted in the biomass by cell divisions. Studies with cycloheximide confirmed that luciferase was relatively stable in 8Dluc cells with a half-life of at least 30 h (data not shown).

3.4. Quantitative control of luciferase expression

Dose–response analysis of luciferase expression levels was examined in cell extracts of 8Dluc cells exposed to Tc concentrations ranging from 100 ng/l to 10 mg/l during 4 days. The Tc concentrations used had no effect on growth rate or cell morphology. Fig. 4 shows that maximal induction was achieved at Tc concentrations of 0.01 mg/l or below. Increasing Tc concentrations resulted in a decrease of the luciferase expression level. Approximately half of the maximal luciferase expression level was measured at a Tc concentration of 0.1 mg/l, while at concentrations of 1 mg Tc/l or higher *tTA*_s* was totally inactivated and luciferase activity was undetectable.

3.5. GFP expression under inducing and non-inducing conditions

FACS analysis of GFP expressing cells enabled us to study the activity of the inducible promoter in individual cells. Three of the *tet* cell lines were transformed with the TRE-P_{min}/*gfp** response plasmid. The GFP fluorescence patterns of fully induced cells (in the absence of Tc) and cells grown under repressing conditions (3 mg Tc/l) were

compared to the fluorescence patterns obtained with various cell lines not expressing GFP (non-transformed *tet* cells and AX3 cells, as well as AX3 cells transformed with the TRE-P_{min}-plasmid MB38). In addition, we examined expression of GFP under the control of A15P in AX3 cells. We determined that the autofluorescence was similar in all cell lines that were not transformed with a *gfp** reporter plasmid, and not influenced by the presence of Tc (data not shown). All non-induced *tet*+TRE-P_{min}/*gfp** cell lines exhibited fluorescence patterns indistinguishable from autofluorescence.

As shown in Table 2, GFP expression under control of A15P in AX3 cells was quite similar in different subclones, with a mean fluorescence of 109–136 AU in only 28–38% of the cells. Apparently, in 62–72% of the cells, GFP expression is not detectable. When *tet*+TRE-P_{min}/*gfp** cells were maximally induced, we also observed that only a portion of the cells showed GFP fluorescence. Among different *tet*+TRE-P_{min}/*gfp** cell lines, the mean GFP fluorescence and the fraction of positive cells ranged from 44 AU in 11% of the cells to 229 AU in 58% of the cells. In this experiment, the strongest *tTA*_s*-regulatable cell line (*tet5*+TRE-P_{min}/*gfp**, clone 5Bgfp) expressed about 3-fold more GFP than cells expressing GFP under the control of A15P.

3.6. Gradual regulation of GFP expression in individual cells

FACS analysis of GFP expression at a range of Tc concentrations was performed, in order to analyze whether partial induction in a cell population at intermediate Tc concentrations was either due to full induction

Fig. 5. FACS analysis of gradual regulation of *gfp** expression. (a) Quantification of mean GFP fluorescence of GFP positive 2Agfp cells (gray bars) and GFP positive 5Dgfp cells (black bars). Following excitation at 488 nm, GFP fluorescence was detected using a 525/20 nm emission filter, and expressed in arbitrary units (AU). (b) Cells from clone 5Dgfp were grown on plates in HG5 medium in the presence of the Tc concentrations indicated (mg/l). On the x-axis, the GFP fluorescence is plotted on a logarithmic scale (emission filter 525/20 nm). Non-specific fluorescence (PE emission filter, 575/30 nm) is plotted on the y-axis on a logarithmic scale. The position of line R2 results from our measurements of the fluorescence in *tet5* cells without reporter plasmid. Cells in the region to the right of line R2 were considered to be GFP positive. Similar results were obtained with 2Agfp cells (data not shown).

in an increasing number of cells, or to a gradual induction in all positive cells. Partial activation of GFP expression was studied by exposing *tet2* and *tet5* cells transformed with TRE-P_{min}/*gfp** (clones 2Agfp and 5Dgfp, respectively) to a wide range of Tc concentrations for 4 days.

Fig. 5a reveals that GFP expression was induced in a dose-dependent manner in individual cells. Only background fluorescence was observed in the presence of 3 or 10 mg/l Tc, while maximal GFP expression was reached at concentrations of 0.03 mg/l or lower. FACS analysis was not sensitive enough to distinguish between the background fluorescence or a low expression level of GFP, in cells grown at 1 mg/l of Tc. The FACS profiles for cells 5Dgfp (Fig. 5b) clearly show that decreasing concentrations of Tc (0.3 or 0.1 mg/l Tc) resulted in a gradual increase of GFP fluorescence in the positive cells (in the region to the right of line R2 in the Fig. 5b). Apparently, Tc did not affect the number of positive cells, since the fraction of positive cells (approx. 60%) remained constant. Similar results were obtained with FACS analysis of cells of clone 2Agfp (data not shown).

4. Discussion

In this report we describe the construction and characterization of a tetracycline-regulated inducible gene expression system in *D. discoideum*, based on a similar system for mammalian cells originally described by Gossen and Bujard (1992).

4.1. Inducible promoter

Transcription is driven by an inducible *D. discoideum* promoter, TRE-P_{min}, through the action of the transactivator protein tTA_s*. The activity of tTA_s* is repressed by Tc. In TRE-P_{min}, the enhancer sequences of the *D. discoideum* A15P (Cohen et al., 1986; Hori and Firtel, 1994) have been replaced by a DNA fragment containing seven *tet* operator sequences. TRE-P_{min} was introduced in an extrachromosomal plasmid, which is stably extrachromosomally maintained under selective conditions. This presents two advantages as compared to the use of an integrated vector. First, TRE-P_{min} is not influenced by endogenous enhancing sequences, so is virtually silent under non-inducing conditions (Table 1). Second, recovery of the response plasmid from transformants is relatively straightforward, which may be useful in mutagenesis studies.

4.2. Analysis of tTA_s*

For efficient induction of TRE-P_{min}, the intracellular concentration of tTA_s* should be sufficiently high. On

the other hand, a relatively high concentration of a VP16 fusion protein, like tTA_s*, is believed to be toxic due to transcriptional squelching effects (Gossen and Bujard, 1992; Shockett and Schatz, 1996). In order to obtain cell lines with optimal induction characteristics, we transformed cells with an integrating vector (Fig. 2) for constitutive expression of tTA_s*. Variation in both the copy number of this transactivator plasmid and the site of chromosomal integration will result in different *tet* cell lines which have varying expression levels of tTA_s*. Seven *tet* cell lines were analyzed. We observed a slight reduction in growth rate in most of the *tet* cell lines, suggesting that expression of tTA_s* may have some negative effect on the transcriptional machinery or physiology of the cell.

The range of luciferase and GFP expression levels from reporter constructs in maximally induced cells varied widely among the various *tet* cell lines (Tables 1 and 2). Moreover, different subclones obtained from the same *tet* cell line displayed some variation in expression levels, which may be a result from variation in the copy number of the reporter plasmid. The maximum levels of luciferase and GFP expression were 1.3-fold and 3-fold higher, respectively, as compared with the levels obtained with A15P. The maximal induction factor of approx. 3000-fold (Table 1) is in the same range as observed for the tTA- based expression systems in mammalian cells (Gossen and Bujard, 1992; Kringstein et al., 1998; Shockett and Schatz, 1996) and yeast (Gari et al., 1997). In summary, our results indicate that tTA_s* is a potent transactivator in *D. discoideum*.

4.3. Kinetics of induction and repression

Inactivation of tTA_s*-regulated transcription is a rapid process. The data in Fig. 3 show that upon addition of Tc (10 mg/l), the *luc** RNA level decreased by 100-fold within 30 min. Since no new protein can be made, we conclude that the slow decrease of luciferase activity in the biomass is due to the relative stability of pre-existing luciferase in *D. discoideum*. In contrast, switching the system on upon removal of Tc is a somewhat slower process than repression (Fig. 3). Half-maximal induction was reached by 8 h (*luc** mRNA) and 18 h (luciferase activity). This result is consistent with the observations in mammalian cells (Gossen and Bujard, 1992; Kringstein et al., 1998), *Drosophila* (Bello et al., 1998) and yeast (Gari et al., 1997). An other version of Tc-regulated systems employs reverse tTA (rtTA), a mutant of tTA requiring the addition of the Tc analog doxycycline to induce binding to the TRE (Gossen et al., 1995). Although it could be expected that the rtTA system would allow for faster induction kinetics, similar kinetics of activation and repression using either tTA- or rtTA-based systems were reported for mammalian cells (Gossen et al., 1995; Kringstein et al., 1998).

Attempts to use rtTA in *Drosophila* (Bello et al., 1998) were unsuccessful, and in yeast repression of the rtTA-system was incomplete (Belli et al., 1998). A different approach to improve the kinetics of induction of the tTA_s-based system in *D. discoideum* might be the use of a Tc-like compound with a short in vivo half-life, or an antagonist of Tc (Chrast-Balz and Hooft van Huijsdijnen, 1996).

4.4. Quantitative regulation

The expression level of luciferase, driven by the inducible promoter TRE-P_{min} in tTA_s expressing cells, was quantitatively controlled by the concentration of Tc (Fig. 4). A dose-dependent response to Tc has also been reported for mammalian cells (Gossen and Bujard, 1992; Kringstein et al., 1998; Zeng et al., 1998) and yeast (Gari et al., 1997). The concentration range (0.01–1 mg/l) of Tc, allowing partial induction in *D. discoideum*, was in agreement with the range observed for yeast but differed markedly from the range typically reported for mammalian cells (0.0001–0.1 mg/l). These different ranges are probably due to species-specific Tc metabolism.

4.5. Regulation at single cell level

FACS analysis allowed us to measure GFP expression in every single *D. discoideum* cell of a clonal population. As shown in Fig. 5, step-wise decreasing the concentration of Tc below 1 mg/l leads to a gradual increase of GFP expression at the individual cell level in GFP positive cells, rather than a gradual increase in the number of cells expressing GFP. This graded response in individual cells is in agreement with data obtained for mammalian cells (Kringstein et al., 1998; Zeng et al., 1998).

However, we noted a marked heterogeneity in GFP expression. Irrespective of whether GFP expression is controlled by A15P or regulated by tTA_s, all clonal populations showed a bimodal distribution of cells, consisting of subpopulations of GFP-negative cells and GFP-positive cells (Table 2). Since the half-life of GFP in *D. discoideum* is relatively long (at least 30 h, our own observations and Deichsel et al., 1999), we conclude that GFP-negative *D. discoideum* cells did not express the reporter gene during several doubling times. Experiments using sorted cells of an AX3 cell line transformed with the A15P/*gfp** plasmid showed that the GFP negative cells did not grow in the presence of blasticidin (data not shown), indicating that in these cells the reporter plasmid is lost. GFP expression in positive cells displayed a 100-fold range of fluorescence intensity. The mechanism for this heterogeneity is still unclear, but it is not specific for this expression system. It has been observed using integrating plasmids in *D.*

discoideum for A15P-controlled expression of GFP (Pang et al., 1999), in *Polysphondylium pallidum* for actin6 promoter (A6P)-controlled expression of GFP (Fey et al., 1995), in *D. discoideum* for A6P-controlled expression of beta-galactosidase (Dingermann et al., 1989) and it has been observed in mammalian cells expressing GFP (Cheng et al., 1996; Klucher et al., 1997).

4.6. Applications

The inducible expression system is particularly useful for several applications. Constructing gene knock-out strains may fail occasionally, which is often interpreted as that the gene is essential. Induced expression of the gene, in the absence of Tc, allows for isolation of a cell line with the disruption of the chromosomal gene; subsequently turning-off expression may provide proof that the gene is essential. Furthermore, the rapid decline of transcription upon addition of Tc allows for the analysis of mRNA and protein stability. An obvious application of the inducible system is the expression of toxic proteins. However, the protein may not be toxic, but merely reduce growth. During the initial isolation of transformants this may result in selection of epigenetic mutations that restore the growth rate. The isolation of stable transformants in the absence of expression prevents the selection of these unwanted mutations. The subsequently turned-on expression provides information on the function of the (toxic) protein.

In conclusion, we have established a tetracycline-regulated gene expression system allowing tight control of reporter gene expression in vegetatively growing *D. discoideum* cells. The system is robust, with no detectable expression in the 'off' state, and the expression can be modulated in a time- and level-controllable fashion. We expect this system to be a valuable tool for studying *Dictyostelium* development and signal transduction, as well as for expression of heterologous proteins.

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